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(54) Title: BIOCATALYTIC METHODS FOR SYNTHESIZING AND IDENTIFYING BIOLOGICALLY ACTIVE COMPOUNDS

(57) Abstract

This invention encompasses methods for producing a library of modified starting compounds by use of biocatalytic reactions on a starting compound and identifying the modified starting compound with the optimum desired activity. The invention encompasses starting compounds and modified compounds that are free in solution. The method is useful in producing modified pharmaceutical compounds with desired specific activity.

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# BIOCATALYTIC METHODS FOR SYNTHESIZING AND IDENTIFYING BIOLOGICALLY ACTIVE COMPOUNDS

## BACKGROUND OF THE INVENTION

(a) Field of the Invention

This invention is in the field of synthesizing and identifying biologically active compounds.

(b) Description of the Prior Art

The prior art is repleat with examples of chemically, microbially, or enzymatically synthesizing compounds with biological activity. The goal of these efforts is the discovery of new and improved pharmaceutical compounds.

The discovery of new pharmaceutical compounds is for the most part a trial and error process. So many diverse factors constitute an effective pharmaceutical compound that it is extremely difficult to reduce the discovery process to a systematic approach. Typically, thousands of organic compounds must be isolated from biological sources or chemically synthesized and tested before a pharmaceutical compound is found.

Synthesizing and testing new compounds for biological activity, which is the first step in identifying a new synthetic drug, is a time consuming and expensive undertaking. Typically, compounds must by synthesized, purified, tested and quantitatively compared to other compounds in order to identify active compounds or identify compounds with optimal activity. The synthesis of new compounds is accomplished for the most part using standard chemical methods. Such methods provide for the synthesis of virtually any type of organic compound; however, because chemical reactions are non-specific, these syntheses require numerous steps and multiple purifications before a final compound is produced and ready for testing.

New biological and chemical approaches have recently been developed which provide for the synthesis and screening of large libraries of small peptides and oligonucleotides. These methods provide for the synthesis of a broad range of chemical compounds and provide the means to potentially identify biologically active compounds. The chemistries for synthesizing such large numbers of these natural and non-naturally occurring polymeric compounds is complicated, but manageable because each compound is synthesized with the same set of chemical protocols, the difference being the random order in which amino acids or nucleotides are introduced into the reaction sequence.

Fodor, S.P.A. et al (1990) Science 251, 767-773, describe methods for discovering new peptide ligands that bind to biological receptors. The process combines solid-phase chemistry and photolithography to achieve a diverse array of small peptides. This work and related works are also described in Fodor WO Patent #9,210,092, Dower WO #9,119,818, Barrett WO #9,107,087 and Pirrung WO#9,015,070.

Houghten, R.A. et al. (1991) Nature 354, 84-86, describe an approach that synthesizes libraries of free peptides along with an iterative selection process that permits the systematic identification of optimal peptide ligands. This work is also described in Appl. WO Patent #9,209,300.

Lam, K.S., et al. (1991) Nature 354, 82-84, describe a method that provides for the systematic synthesis and screening of peptide libraries on a solid-phase microparticle support on the basis of a 'one-bead, one-peptide' approach.

Cwirla, S.E., et al (1990) Proc. Natl. Acad. Sci. USA 87, 6378-6382, describe a method for constructing a library of peptides on the surface of a phage by cloning randomly synthesized oligonucleotides into the 5' region of specific phage genes resulting in millions of different hexapeptides expressed at the N terminus of surface proteins.

These methods accelerate the identification of biologically active peptides and oligonucleotides. However, peptides and oligonucleotides have poor bioavailability and limited stability in vivo, which limits their use as therapeutic agents. In general, non-biological compounds which mimic the structure of the active peptides and oligonucleotides must be synthesized based on the approximated three dimensional structure of the peptide or oligonucleotide and tested before an effective drug structure can be identified.

Bunin et al., J. Am. Chem. Soc. (1992) 114, 10997-10998 describe the synthesis of numerous 1,4 benzodiazapine derivatives using solid phase synthesis techniques.

The prior art is repleat with examples showing enzymatic conversion of non-physiological substances under many conditions.

**References demonstrating that enzyme specificity can be changed/tailored:**

1. Zaks, A. and Klibanov, A.M. Substrate specificity of enzymes in organic solvents vs. water is reversed. Journal of the American Chemical Society 108 2767-2768, 1986.

2. Ferjancic, A., Puigserver, A. and Gaertner, H. Unusual specificity of PEG-modified thermolysin in peptide synthesis catalyzed in organic solvents. Biotechnology Letters 10 (2) 101-106, 1988.

3. Nasri, M. and Thomas, D. Increase of the potentialities of restriction endonuleases by specificity relaxation in the presence of organic solvents. Ann. N.Y. Acad. Sci. 542 255-265, 1988.

4. Stahl, M., Mansson, M.O. and Mosbach, K. The synthesis of a D-amino acid ester in an organic media with chymotrypsin modified by a bio-imprinting procedure. Biotechnology Letters 12 (3) 161-166, 1990.

5. Ståhl, M., Jeppsson-Wistrand, U., Mansson, M.O. and Mosbach, K. Induced stereoselectivity and substrate selectivity of bio-imprinted a-chymotrypsin in anhydrous organic media. Journal of the American Chemical Society 113 (24) 9366-9368, 1991.

6. Gololobov, M.Y., Voyushina, T.L., Stepanov, V.M. and Adlercreutz, P. Organic solvent changes the chymotrypsin specificity with respect to nucleophiles. FEBS Letters 307 (3) 309-312, 1992.

7. Hertmanni, P., Pourplanche, C. and Larreta-Garde, V. Orientation of enzyme catalysis and specificity by water-soluble additives. Ann. New York Acad.Sci. (Enzyme Eng. XI, D.S. Clark, D.A. Estell, eds) 672 329-335, 1992.

8. Cabezas, M.J., del Campo, C., Llama, E., Sinisterra, J.V. and Gaertner, H. Organic reactions catalysed by modified enzymes. 1. Alteration of the substrate specificity of a-chymotrypsin by the modification process. Journal of Molecular Catalysis 71 (2) 261-278, 1992.

9. Nagashima, T., Watanabe, A. and Kise, H. Peptide synthesis by proteases in organic solvents: medium effect on substrate specificity. Enzyme and Microbial Technology 14 (10) 842-847, 1992.

10. Parida, S. and Dordick, J.S. Tailoring lipase specificity by solvent and substrate chemistries, J. Org. Chem. 58 (12) 3238-3244, 1993.

11. Tawaki, S. and Klibanov, A.M. Chemoselectivity of enzymes in anhydrous media is strongly solvent dependent. Biocatalysis 8 (1) 3-19, 1993.

12. Wescott, C.F. and Klibanov, A.M. Solvent variation inverts substrate specificity of an enzyme. JACS 115 (5) 1629-1631, 1993.

**References demonstrating that enzyme enantioselectivity can be changed/tailored:**

1. Sakurai, T., Margolin, A.L., Russell, A.J. and Klibanov, A. M. Control of enzyme enantioselectivity by the reaction medium. Journal of the American Chemical Society 110 (21) 7236-7237, 1988.

2. Fitzpatrick, P.A. and Klibanov, A.M. How can the solvent affect enzyme enantioselectivity? Journal of the American Chemical Society 113 (8) 3166-3171, 1991.

3. Hult, K. and Norin, T. Enantioselectivity of some lipases - control and prediction. Pure and Applied Chemistry 64 (8) 1129-1134, 1992.

4. Miyazawa, T., Kurita, S., Ueji, S., Yamada, T. and Kuwata, S. Resolution of racemic carboxylic acids via the lipase-catalyzed irreversible transesterification using vinyl esters - effects of alcohols as nucleophiles and organic solvents on enantioselectivity. Biotechnology Letters 14 (10) 941-946, 1992.

5. Tawaki, S. and Klibanov, A.M. Inversion of enzyme enantioselectivity mediated by the solvent. Journal of the American Chemical Society 114 (5) 1882-1884, 1992.

6. Ueji, S., Fujino, R., Okubo, N., Miyazawa, T., Kurita, S., Kitadani, M. and Muromatsu, A. Solvent-induced inversion of enantioselectivity in lipase-catalyzed esterification of 2-phenoxypropionic acids. Biotechnology Letters 14 (3) 163-168, 1992.

7. Terradas, F., Testonhenry, M., Fitzpatrick, P.A. and Klibanov, A.M. Marked dependence of enzyme prochiral selectivity on the solvent. Journal of the American Chemical Society 115 (2) 390-396, 1993.

8. Herradon, B. Biocatalytic synthesis of chiral polyoxygenated compounds: effect of the solvent on the enantioselectivity of lipase catalyzed transesterifications in organic solvents. Synlett 2 108-110, 1993.

**References demonstrating the ability of enzymes to convert unnatural substrates:**

1. Bianchi, D., Cesti, P., Golini, P., Spezia, S., Garavaglia, C. and Mirenna, L. Enzymatic preparation of optically active fungicide intermediates in aqueous and in organic media. Pure and Applied Chemistry 64 (8) 1073-1078, 1992.

2. Natoli, M., Nicolosi, G. and Piattelli, M. Regioselective alcoholysis of flavonoid acetates with lipase in an organic solvent. Journal of Organic Chemistry 57 (21) 5776-5778, 1992.

3. Izumi, T., Tamura, F. and Sasaki, K. Enzymatic kinetic resolution of <4>(1,2)ferrocenophane derivatives. Bulletin of the Chemical Society of Japan 65 (10) 2784-2788, 1992.

4. Miyazawa, T., Mio, M., Watanabe, Y., Yamada, T. and Kuwata, S. Lipase-catalyzed transesterification procedure for the resolution of non-protein amino acids. Biotechnology Letters 14 (9) 789-794, 1992.

5. Murata, M., Uchida, H. and Achiwa, K. Lipase-catalyzed enantioselective synthesis of optically active mephobarbital, hexobarbital and febarbamate. Chemical-Pharmaceutical Bulletin 40 (10) 2605-2609, 1992.

6. Johnson, C.R., Golebiowski, A. and Steensma, D.H. Enzymatic asymmetrization in organic media - synthesis of unnatural glucose from cycloheptatriene. Journal of the American Chemical Society 114 (24) 9414-9418, 1992.

7. Cruces, M.A., Otero, C., Bernabe, M., Martinlomas, M. and Ballesteros, A. Enzymatic preparation of acylated sucroses. Ann. New York Acad.Sci. (Enzyme Eng. XI, D.S. Clark, D.A. Estell, eds) 672 436-443, 1992.

8. Tanaka, A., Fukui, T., Uejima, A., Zong, M.H. and Kawamoto, T. Bioconversion of nonnatural organic compounds - esterification and dehydrogenation of organosilicon compounds. Ann. New York Acad. Sci (Enzyme Eng. XI, D.S. Clark, D.A. Estell, eds) 672 431-435, 1992.

9. Kodelia, G. and Kolisis, F.N. Studies on the reaction catalyzed by protease for the acylation of flavonoids in organic solvents. Ann. New York Acad.Sci (Enzyme Eng. XI, D.S. Clark, D.A. Estell, eds) 672 451-457, 1992.

10. Wagner, F., Kleppe, F., Lokotsch, W., Ziemann, A. and Lang, S. Synthesis of uncommon wax esters with immobilized lipases. Ann. New York Acad.Sci. (Enzyme Eng. XI, D.S. Clark, D.A. Estell, eds) 672 484-491, 1992.

11. Patel. R.N., Howell, J.M., Banerjee, A., Fortney, K.F. and Szarka, L. J. Stereoselective enzymatic esterification of 3-benzoylthio-2-methylpropanoic acid. Ann. New York Acad. Sci (Enzyme Eng. XI, D.S. Clark, D.A. Estell, eds) 672 415-424, 1992.

12. Bergbreiter, D.E. and Momongan, M. Asymmetric synthesis of organometallic reagents using enzymatic methods. Applied Biochemistry and Biotechnology 32 1-3 55-72, 1992.

13. Carretero, J.C. and Dominguez, E. Lipase-catalyzed kinetic resolution of _-hydroxy phenyl sulfones. Journal of Organic Chemistry 57 (14) 3867-3873, 1992.

14. Johnson, C.R., Adams, J.P., Bis, S.J., Dejong, R.L., Golebiowski, A., Medich, J. R., Penning, T.D., Senanayake, C.H., Steensma, D.H. and Vanzandt, M.C. Applications of enzymes in the synthesis of bioactive polyols. Indian Journal of Chemistry Section B - Organic Chemistry Including Medicinal Chemistry 32 (1) 140-144, 1993.

15. Fabre, J., Betbeder, D., Paul, F., Monsan, P. and Perie, J. Regiospecific enzymic acylation of butyl a-D-glucopyranoside. Carbohydrate Research 243 (2) 407-411, 1993.

16. Bianchi, D., Cesti, P., Golini, P., Spezia, S., Garavaglia, C. and Mirenna, L. Enzymatic preparation of optically active fungicide intermediates in aqueous and in organic media. Indian Journal of Chemistry Section B - Organic Chemistry Including Medicinal Chemistry 32 (1) 176-180, 1993.

17. Kanerva, L.T. and Sundholm, O. Lipase catalysis in the resolution of racemic intermediates of diltiazem synthesis in organic solvents. Journal of the Chemical Society - Perkin Transactions I 13 1385-1389, 1993.

18. Ikeda, I. and Klibanov, A.M. Lipase-catalyzed acylation of sugars solubilized in hydrophobic solvents by complexation. Biotechnology and Bioengineering 42(6) 788-791, 1993.

19. Hyun, C.K., Kim, J.H. and Ryu, D.D.Y. Enhancement effect of water activity on enzymatic synthesis of cephalexin. Biotechnology and Bioengineering 42(7) 800-806, 1993.

20. Panza, L., Luisetti, M., Crociati, E. and Riva, S. Selective acylation of 4,6-O-benzylidene glycopyranosides by enzymatic catalysis. J. Carbohydrate Chem. 12 (1) 125-130, 1993.

21. Wang, L., Kobatake, E., Ikariyama, Y. and Aizawa, M. Regioselective oxidative polymerization of 1,5- dihydroxynaphthalene catalyzed by bilirubin oxidase in a water-organic solvent mixed solution. Journal of Polymer Science Part A - Polymer Chemistry 31 (11) 2855-2861, 1993.

22. Knani, D. and Kohn, D.H. Enzymatic polyesterification in organic media. 2. Enzyme-catalyzed synthesis of lateral-substituted aliphatic polyesters and copolyesters. J. Polymer Sci., Part A - Polymer Chem. 31 (12) 2887-2897, 1993.

23. Kanerva, L.T. and Sundholm O. Enzymatic acylation in the resolution of methyl threo-2-hydroxy-3-(4-methoxyphenyl)-3-(2-x-phenylthio) propionates in organic solvents. Journal of the Chemical Society - Perkin Transactions I 20 2407-2410, 1993.

24. Sharma, A. and Chattopadhyay, S. Lipase catalysed acetylation of carbohydrates. Biotechnology Letters 15 (11) 1145-1146, 1993.

25. Pavel, K. and Ritter, H. Enzymes in polymer chemistry. 7. Lipase-catalyzed esterification of carboxyl-terminated methacrylic oligomers and copolymers with isopropyl alcohol

and 9-fluorenylmethanol. Makromolekulare Chemie- Macromolecular Chemistry and Physics 194 (12) 3369-3376, 1993.

26. Uejima, A., Fukui, T., Fukusaki, E., Omata, T., Kawamoto, T., Sonomoto, K. and Tanaka, A. Efficient kinetic resolution of organosilicon compounds by stereoselective esterification with hydrolases in organic solvent. Appl. Microbiol.Biotech. 38 (4) 482-486, 1993.

27. Mukesh, D., Sheth, D., Mokashi, A., Wagh, J., Tilak, J.M., Banerji, A.A. and Thakkar, K.R. Lipase catalysed esterification of isosorbide and sorbitol. Biotechnology Letters 15 (12) 1243-1246, 1993.

28. Baldessari, A., Iglesias, L.E. and Gros, E.G. Regioselective acylation of 3-mercaptopropane-1,2-diol by lipase-catalysed transesterification. Journal of Chemical Research - S 9 382-383, 1993.

29. De Goede, A.T.J.W., Benckhuijsen, W., van Rantwijk, F., Maat, L. and van Bekkum, H. Selective lipase-catalyzed 6-O-acylation of alkyl a-D-glucopyranosides using functionalized ethyl esters. Recueil Des Travaux Chimiques Des Pays Bas - Journal of the Royal Netherlands Chemical Society 112 (11) 567-572, 1993.

30. Menendez,E. and Gotor, V. Acylation and alkoxycarbonylation of oximes through an enzymatic oximolysis reaction. Synthesis - Stuttgart 1 72-74, 1993.

31. Li, Y.F. and Hammerschmidt, F. Enzymes in organic chemistry. 1. Enantioselective hydrolysis of a-(acyloxy) phosphonates by esterolytic enzymes. Tetrahedron - Asymmetry 4 (1) 109-120, 1993.

32. Schlotterbeck, A., Lang, S., Wray, V. and Wagner, F. Lipase-catalyzed monoacylation of fructose. Biotechnology Letters 15 (1) 61-64, 1993.

33. Frykman, H., Ohrner, N., Norin, T. and Hult, K. S-Ethyl thiooctanoate as acyl donor in lipase catalysed resolution of secondary alcohols. Tetrahedron Letters 34 (8) 1367-1370, 1993.

34. Oguntimein, G.B., Erdmann, H. and Schmid, R.D. Lipase catalysed synthesis of sugar ester in organic solvents. Biotechnology Letters 15 (2) 175-180, 1993.

35. Lopez, R., Perez, C., Fernandez-Mayorales, A. and Conde, S. Enzymatic transesterification of alkyl 2,3,4-tri-O-acyl-beta-D-xylopyranosides. J. Carbohydrate Chem. 12 (2) 165-171, 1993.

36. Naemura, K., Ida. H. and Fukuda, R. Lipase YS-catalyzed enantioselective transesterification of alcohols of bicarbocyclic compounds. Bull. Chem. Soc.Japan 66 (2) 573-577, 1993.

37. Lambusta, D., Nicolosi, G., Piattelli, M. and Sanfilippo, C. Lipase catalyzed acylation of phenols in organic solvents. Ind. J .Chem. Section B 32 (1) 58-60, 1993.

38. Astorga, C., Rebolledo, F. and Gotor, V. Enzymatic hydrazinolysis of diesters and synthesis of N-aminosuccinimide derivatives. Synthesis - Stuttgart 3 287-289, 1993.

39. Fukui, T., Kawamoto, T. and Tanaka, A. Enzymatic preparation of optically active silylmethanol derivatives having a stereogenic silicon atom by hydrolase-catalyzed enantioselective esterification. Tetrahedron - Asymmetry 5 (1) 73-82, 1994.

40. Vazquez-Duhalt, R., Westlake, D.W.S. and Fedorak, P.M. Lignin peroxidase oxidation of aromatic compounds in systems containing organic solvents. Applied and Environmental Microbiology 60 (2) 459-466, 1994.

41. Kodelia, G., Athanasiou, K. and Kolisis, F.N. Enzymatic synthesis of butyryl-rutin ester in organic solvents and its cytogenetic effects in mammalian cells in culture. Appl. Biochem. Biotech. 44 (3) 205-212, 1994.

42. Tsai, S.W. and Wei, H.J. Effect of solvent on enantioselective esterification of naproxen by lipase with trimethylsilyl methanol. Biotechnology and Bioengineering 43 (1) 64-68, 1994.

43. Athawale, V.D. and Gaonkar, S.R. Enzymatic synthesis of polyesters by lipase catalysed polytransesterification. Biotechnology Letters 16 (2) 149-154, 1994.

44. Janssen, G.G. and Haas, M.J. Lipase-catalyzed synthesis of oleic acid esters of polyethylene glycol 400. Biotechnology Letters 16 (2) 163-168, 1994.

45. Kitazuma, T., Ikeya, T. and Murata, K. Synthesis of optically active trifluorinated compounds: asymmetric Michael additional with hydrolytic enzymes. Journal of Chemical Society, Chemical Communications 1331, 1986.

**References demonstrating microbial transformations:**

1. Holland, H.L. Fungi as reagents in organic synthesis: preparation of some metabolites of prostanoids, steroids, and other natural products. Rev. Latinoam. Quim., (1st Spec. Suppl.), 318-329, 1990.

2. Sih, C.J. and Rosazza, J.P. Microbial transformations in organic synthesis. Tech. Chem. (N.Y.), 10 (Appl. Biochem. Syst. Org. Chem., Part 1) 69-106, 1976.

3. Sih, C.J. and Chen, C.S. Microbial asymmetric catalysis-enantioselective reduction of ketones. Angew. Chem. Int. Ed. Engl. 23 (8) 570-578, 1984.

4. Thompson, L.A. and Knowles, C.J., Linton, E.A. and Wyatt, J.M. Microbial biotransformations of nitriles. Chem. Br. 24 (9) 900, 1988.

5. Servi, S. Baker's yeast as a reagent in organic synthesis. Synthesis 1 1-25, 1990.

6. Csuk, R. and Glanzer, B.I. Baker's yeast mediated transformations in organic chemistry. Chem. Rev. 91 (1) 49-97, 1991.

7. Roberts, S.M., Wiggins, K. and Casy, G. *Whole cells and isolated enzymes in organic synthesis.* Wiley, New York, 1992.

8. Ward, O.P. and Young, C.S. Reductive biotransformations of organic compounds by cells or enzymes of yeast. Enxyme Microb. Technol. 12 (7) 482-493, 1990.

**Reviews:**

1. Jones, J.B. Enzymes in organic synthesis. Tetrahedron 42 (13) 3351-3405, 1986.

2. Yamada, H. and Shimizu, S. Microbial and enzymatic processes for the production of biologically and chemically useful compounds. Angew. Chem. Int. Ed. Engl. 27 (5) 622-642, 1988.

3. Roberts, S.M. Enzymes as catalysts in organic synthesis. NATO ASI Ser., Ser. A. 178 443-463, 1989.

4. Chen, S.S. and Sih, C.J. General aspects and optimization of enantioselective biocatalysis in organic solvents: the use of lipases. Angew. Chem (Int. Ed. Engl. 28, n 6 695-707) 101 (6) 711-724, 1989.

## SUMMARY OF THE INVENTION

The present invention is used to synthesize a library of non-biological organic compounds from a starting compound and identify individual compounds within the library which exhibit biological activity. Unlike peptides and oligonucleotides, non-biological organic compounds comprise the bulk of proven therapeutic agents. The invention can be used to directly identify new drug candidates or optimize an established drug compound which has sub-optimal activity or problematic side effects. This is accomplished through the use of highly specific biocatalytic reactions.

Enzymes are highly selective catalysts. Their hallmark is the ability to catalyze reactions with exquisite stereo-, regio-, and chemo-selectivities that are unparalleled in conventional synthetic chemistry. Moreover, enzymes are remarkably versatile. They can be tailored to function in organic solvents, operate at extreme pH's and temperatures, and catalyze reactions with compounds that are structurally unrelated to their natural, physiological substrates.

Enzymes are reactive toward a wide range of natural and unnatural substrates, thus enabling the modification of virtually any organic lead compound. Moreover, unlike traditional chemical catalysts, enzymes are highly enantio- and regio-selective. The high degree of functional group specificity exhibited by enzymes enables one to keep track of each reaction in a synthetic sequence leading to a new active compound. Enzymes are also capable of catalyzing many diverse reactions unrelated to their physiological function in nature. For example, peroxidases catalyze the oxidation of phenols by hydrogen peroxide. Peroxidases can also catalyze hydroxylation reactions that are not related to the native function of the enzyme. Other examples are proteases which catalyze the breakdown of polypeptides. In organic solution some proteases can also acylate sugars, a function unrelated to the native function of these enzymes.

The present invention exploits the unique catalytic properties of enzymes. Whereas the use of biocatalysts (i.e., purified or crude enzymes, non-living or living cells) in chemical transformations normally requires the identification of a particular biocatalyst that reacts with a specific starting compound, the present invention uses selected biocatalysts and reaction conditions that are specific for functional groups that are present in many starting compounds.

Each biocatalyst is specific for one functional group, or several related functional groups, and can react with many starting compounds containing this functional group.

The biocatalytic reactions produce a population of derivatives from a single starting compound. These derivatives can be subjected to another round of biocatalytic reactions to produce a second population of derivative compounds. Thousands of variations of the original compound can be produced with each iteration of biocatalytic derivatization.

Enzymes react at specific sites of a starting compound without affecting the rest of the molecule, a process which is very difficult to achieve using traditional chemical methods. This high degree of biocatalytic specificity provides the means to identify a single active compound within the library. The library is characterized by the series of biocatalytic reactions used to produce it, a so called "biosynthetic history". Screening the library for biological activities and tracing the biosynthetic history identifies the specific reaction sequence producing the active compound. The reaction sequence is repeated and the structure of the synthesized compound determined. This mode of identification, unlike other synthesis and screening approaches, does not require immobilization technologies, and compounds can be synthesized and tested free in solution using virtually any type of screening assay. It is important to note, that the high degree of specificity of enzyme reactions on functional groups allows for the "tracking" of specific enzymatic reactions that make up the biocatalytically produced library.

Many of the procedural steps are performed using robotic automation enabling the execution of many thousands of biocatalytic reactions and screening assays per day as well as ensuring a high level of accuracy and reproducibility. As a result, a library of derivative compounds can be produced in a matter of weeks which would take years to produce using current chemical methods.

The present invention is unique in that it involves a soluble state of the starting compound and its subsequent derivatives. This is a highly unique aspect of the present invention that has been thought to be a barrier. Previous organic modifying technologies for biologically active compound identification involve starting compounds and derivatives attached to insoluble supports. This is taught in examples by Ellman (Bunin, B.S.; Ellman, J.A. "A general and expedient method for the solid-phase synthesis of 1,4-benzodiazepine derivatives", J. Am. Chem. Soc. 1992, 114 10997-10998), Gordon et al., (Gordon, E.M.; Barrett, R.W.; Dower, W.J.; Fodor, S.P.A.; Gallop, M.A. "Applications of Combinatorial Technologies to Drug Discovery, 1. Background and Peptide Combinatorial Libraries", J. Med. Chem. 1994, 37 1233-1251; and Gordon, E.M.; Barrett, R.W.; Dower, W.J.; Fodor, S.P.A.; Gallop, M.A. "Applications of Combinatorial Technologies to Drug Discovery, 2. "Combinatorial Organic Synthesis, Library Screening Strategies, and Future Directions", J. Med. Chem. 1994, 37 1385-1401), Hobbs Dewitt et al., (Hobbs Dewitt, S.; Kiely, J.S.; Stankovic, C.J.; Schroeder, M.C.; Reynolds Cody, D.M.; Pavia, M.R. "Diversomers: An approach to nonpeptide, nonoligomeric chemical diversity" P.N.A.S. (USA) 1993 90 6909-6913). In addition to synthesising the libraries on a soluble state, the libraries of the instant invention can then be screened immediately without removal of the

libraries from solid supports. Thus by maintaining the components in the soluble state, the present invention provides for useful, convienent, and efficient methods of generating and screening libraries of starting compounds and derivatives.

The present invention specifically incorporates a number of diverse technologies such as: (1) the use of enzymatic reactions to produce a library of drug candidates; (2) the use of enzymes free in solution or immobilized on the surface of particles, and organic compounds derivatized while dissolved in solution; (3) the use of receptors (hereinafter this term is used to indicate true receptors, enzymes, antibodies and other biomolecules which exhibit affinity toward biological compounds, and other binding molecules to identify a promising drug candidate within a library, even where such receptors are still associated with cell membranes, or intact cells); (4) the automation of all biocatalytic processes and many of the procedural steps used to test the libraries for desired activities, and (5) the coupling of biocatalytic reactions with drug screening devices which can immediately measure the binding of synthesized compounds to receptor molecules or in whole-cell assays and thereby immediately identify specific reaction sequences giving rise to biologically active compounds.

Specifically, the present invention encompasses a method for drug identification comprising:

(a) conducting a series of biocatalytic reactions by mixing biocatalysts with a starting compound to produce a reaction mixture and thereafter a library of modified starting compounds;

(b) testing the library of modified starting compounds to determine if a modified starting compound is present within the library which exhibits a desired activity;

(c) identifying the specific biocatalytic reactions which produce the modified starting compound of desired activity by systematically eliminating each of the biocatalytic reactions used to produce a portion of the library and testing the compounds produced in the portion of the library for the presence or absence of the modified starting compound with the desired activity; and

(d) repeating the specific biocatalytic reactions which produce the modified compound of desired activity and determining the chemical composition of the reaction product.

More specifically, the enzymatic reactions are conducted with a group of enzymes that react with distinct structural moieties found within the structure of a starting compound. Each enzyme is specific for one structural moiety or a group of related structural moieties. Furthermore, each enzyme reacts with many different starting compounds which contain the distinct structural moiety. In addition to systematically eliminating each reaction, the instant invention also provides for the systematic process of building up each reaction, an approach from the other end of the pathway. A particular feature of the instant invention is the soluble state of the starting compounds and the subsequent derivatives.

## BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the starting active compound AZT with four potential sites for biocatalytic derivatization and eight possible biocatalytic reactions that can be used to produce a library of derivative compounds.

Figure 2 shows an automated system employing robotic automation to perform hundreds of biocatalytic reactions and screening assays per day.

Figure 3 illustrates the tracking of biocatalytic reactions to identify the sequence of reactions producing an active compound, which can subsequently be used to produce and identify the structure of the active compound.

Figure 4a illustrates biocatalytic modification of castanospermine.

Figure 4b illustrates biocatalytic modifications of methotrexate.

## DETAILED DESCRIPTION OF THE INVENTION

While the invention will be described in connection with certain preferred embodiments, it will be understood that the description does not limit the invention to these particular embodiments. The embodiements of the invention are described with AZT, and further described for taxol, only as particular embodiments of the instant invention. In fact, it is to be understood that all alternatives, modifications and equivalents are included and are protected, consistent with the spirit and scope of the inventions as defined in the appended claims.

The preferred embodiments of the invention are set forth in the following example:

a) A starting compound such as AZT (3'-azidothymidine), is chosen which exhibits drug activity or is believed to exhibit drug activity for a given disease or disorder. The compound is analyzed with respect to its functional group content and its potential for structural modifications using selected biocatalytic reactions. Functional groups which can be chemically modified using the selected biocatalytic reactions are listed in Table I. One of more of these functional groups are present in virtually all organic compounds. A partial list of possible enzymatic reactions that can be used to modify these functional groups is presented in Table II.

b) A strategy is developed to systematically modify these functional groups using selected biocatalytic reactions and produce a library of derivative compounds to be screened for biological activity. AZT contains four functional groups which are selected for biocatalytic modification: a primary hydroxyl, two carbonyls and a tertiary amine.

The biosynthetic strategy is designated in the form of biocatalytic "reaction box" numbers which correspond to specific types of biocatalytic reactions acting on specific functional groups present in the starting compound. These "reaction boxes" are listed in Table III. The following biocatalytic

"reaction boxes" are selected to synthesize an AZT derivative library: A3, A10, A11, C2, G6, G10 and G12. FIG. 1 illustrates the reaction of AZT with these selected biocatalytic reaction boxes.

c) The biocatalytic reaction boxes are entered into an automated system which is shown in FIG 2. The system is programmed to automatically execute the aforementioned biocatalytic reactions and synthesize a library of derivative products. A single automated system in capable of performing hundreds of pre-programmed biocatalytic reactions per day. We can estimate the total number of compounds that can be produced by analyzing the reaction products produced in each "reaction box" and multiplying the results. Table IV details the number of potential reaction products produced in each reaction box and the resulting total number of possible compounds produced. In the case of AZT, up to $1.75 \times 10^{11}$ new compounds can be synthesized. It should be pointed out that this compares very favorably to peptide libraries. For example, a library of hexapeptides will contain $20^6$ or 64 million compounds. This is a mere fraction, about 0.04% of the compounds that are possible using the biosynthetic approach described herein. Table V lists the results of a similar analysis on eleven other starting drug compounds. As shown in this table, the biocatalytic reactions can generate huge numbers of derivative compounds for drug screening.

d) The synthesized library of new compounds is assayed using enzyme inhibition assays, receptor- binding assays, immunoassays, and/or cellular assays to identify biologically active compounds. Before assaying the library of derivative compounds, any remaining AZT present in the library is either removed or inhibited to simplify the interpretation of screening assay results. This is easily accomplished by HPLC, TLC, or the addition of a monoclonal antibody specific for the starting compound. Numerous in vitro assays are available that test for anti-viral, anti-cancer, anti-hypertensive and other well known pharmacological activities. Some of these assays are listed in Table VI. Most of these assays are also performed on the automated system.

e) Libraries which test positive are further analyzed using a biocatalytic tracking protocol which quickly identifies the specific sequence of reactions responsible for the synthesis of the compound testing positive in the screening assay. The high degree of specificity exhibited by biocatalysts, i.e., the ability of a given enzyme to react with a given functional group, enables this approach to be easily performed. The library is characterized by the series of biocatalytic reactions used to produce it, a so called "biosynthetic history". Portions of the library are screened for biological

activity until the specific reaction sequence producing the active compound is identified. FIG. 3 illustrates this tracking process. For example, the dark line path 15 illustrates the reaction pathway to the most active compound. The reaction sequence is repeated to produce a sufficient amount of product for chemical analysis. The specificity of the biocatalytic reactions also permits the accurate duplication of the reaction pathway producing the active compounds. The structure of the active compound is qualitatively determined by analyzing the starting compounds, substrates and identified biocatalytic reaction sequence. The structure is then confirmed using gas chromotography, mass spectroscopy, NMR spectroscopy and other organic analytical methods. This mode of identification eliminates the need for product purification and also reduces the amount of test screening required to identify a promising new drug compound. This process dramatically reduces the time necessary to synthesize and identify new drug compounds. In addition, this mode of active compound identification does not require immobilization technologies, and compounds can be synthesized and tested free in solution under in vivo like conditions using virtually any type of screening assay (receptor, enzyme inhibition, immunoassay, cellular, animal model).

Those skilled in the pharmaceutical arts will recognize the large number of biocatalytic conversions such as those listed in Table II and Table III, as well as the in vitro drug screening assays listed in Table VI.

Those skilled in the pharmaceutical arts will recognize that biocatalytic reactions are optimized by controlling or adjusting such factors as solvent, buffer, pH, ionic strength, reagent concentration and temperature.

The biocatalysts used in the biocatalytic reactions may be crude or purified enzymes, cellular lysate preparations, partially purified lysate preparations, living cells or intact non-living cells, used in solution, in suspension, or immobilized on magnetic or non-magnetic surfaces.

In addition, non-specific chemical reactions may also be used in conjunction with the biocatalytic reaction to obtain the library of modified starting compounds. Examples of such non-specific chemical reactions include: hydroxylation of aromatics and aliphatics; oxidation reactions; reduction reactions; hydration reactions; dehydration reactions; hydrolysis reactions; acid/based catalyzed esterification; transesterification; aldol condensation; reductive amination; amminolysis; dehydrohalogenation; halogenation; acylation; acyl substitution; aromatic substitution; Grignard synthesis; Friedel-Crafts acylation; etherification.

The biocatalytic reaction can be performed with a biocatalyst immobilized to magnetic particles forming a magnetic biocatalyst. The method of this embodiment is performed by initiating the biocatalytic reaction by combining the immobilized biocatalyst with substrate(s), cofactors(s) and solvent/buffer conditions used for a specific biocatalytic reaction. The magnetic

biocatalyst is removed from the biocatalytic reaction mixture to terminate the biocatalytic reaction. This is accomplished by applying an external magnetic field causing the magnetic particles with the immobilized biocatalyst to be attracted to and concentrate at the source of the magnetic field, thus effectively separating the magnetic biocatalyst from the bulk of the biocatalyst reaction mixture. This allows for the transferral of the reaction mixture minus the magnetic biocatalyst from a first reaction vessel to a second reaction vessel, leaving the magnetic biocatalyst in the first reaction vessel. A second biocatalytic reaction is conducted completely independent of the first biocatalytic reaction, by adding a second biocatalyst immobilized to magnetic particles to the second reaction vessel containing the biocatalytic reaction mixture transferred from the first reaction vessel. Finally, these steps are repeated to accomplish a sequential series of distinct and independent biocatalytic reactions, producing a corresponding series of modified starting compounds.

The biocatalytic reactions can also be performed using biocatalysts immobilized on any surface which provides for the convenient addition and removal of biocatalyst from the biocatalytic reaction mixture thus accomplishing a sequential series of distinct and independent biocatalytic reactions producing a series of modified starting compounds.

The biocatalytic reactions can also be used to derivatize known drug compounds producing new derivatives of the drug compound and select individual compounds within this library that exhibit optimal activity. This is accomplished by the integration of a high affinity receptor into the biocatalytic reaction mixture, which is possible because of the compatibility of the reaction conditions used in biosynthesis and screening. The high affinity receptor is added to the reaction mixture at approximately one half the molar concentration of the starting active compound, resulting in essentially all of the receptor being bound with the starting active compound and an equal molar concentration of starting active compound free in solution and available for biocatalytic modification. If the biocatalytic reaction mixture produces a derivative which possesses a higher binding affinity for the receptor, which can translate into improved pharmacological performance, this derivative will displace the bound starting active compound and remain complexed with the receptor, and thus be protected from further biocatalytic conversions. At the end of the experiment, the receptor complex is isolated, dissociated and the bound compound analyzed. This approach accomplishes the identification of an improved version of the drug compound without the need to purify and test each compound individually.

The biocatalytic reactions and in vitro screening assays can be performed with the use of an automated robotic device. The automated robotic device having:

(a) an XY table with an attached XYZ pipetting boom to add volumetric amounts of enzyme, substrate, cofactor, solvent solutions and assay reagents from reagent vessels positioned on the XY table to reaction and assay vessels positioned on the same XY table;

(b) an XYZ reaction-vessel transfer boom attached to the same XY table used to transfer reaction and assay vessels positioned on the XY table to different locations on the XY table;

(c) a temperature incubation block attached to the same XY table to house the reaction and assay vessels during reaction incubations and control the temperature of the reaction mixtures;

(d) a magnetic separation block attached to the same XY table to separate the biocatalyst immobilized to magnetic particles from the biocatalytic mixture by applying an external magnetic field causing the magnetic particles to be attracted to and concentrate at the source of the magnetic filed, thus effectively separating them from the bulk of the biocatalytic reaction mixture; and

(e) a programmable microprocessor interfaced to the XYZ pipetting boom, and XYZ reaction-vessel transfer boom, the temperature block and the magnetic separation block to precisely control and regulate all movements and operations of these functional units in performing biocatalytic reactions to produce modified starting compounds and assays to determine desired activities.

Figure 2 illustrates the automated robotic device of this invention. Mounted in the frame 1 of the system are containers for starting compounds 2, and containers for reagents 3 such as enzymes, cofactors, and buffers. There are specific biosynthesis boxes 4 which contain reagents for various classes of reactions. The frame also has arrays of reaction vessels 5, and a heating block 6 with wells 7 for conducting reactions at a specific temperature. The frame has an area 8 for reagents for screening test 8 which contains reagents used for conducting screening tests, and area 9 which contains assay vessels for conducting screening tests, the automated system uses a X-Y-Z pipetting and vessel transfer boom 10 to dispense all reagents and solutions, and transfer reaction vessels.

In operation the X-Y-Z reaction-vessels transfer boom can deliver starting compounds and reagents to specific locations for making specific modified starting compounds which in turn can be delivered to specific locations for conducting assays. In this way the process of making modified starting compounds and testing for optimum activity is largely automated.

Figures 4a and 4b illustrate derivatization of castanospermine and methotrexate. All of these embodiments utilize the biocatalytic conversions set out in Table II and the assays set out in Table VI.

While the invention as described herein is directed to the development of drugs, those skilled in the biological arts will recognize that the methods of this invention are equally applicable to other biologically active compounds such as food additives, pesticides, herbicides, and plant and animal growth hormones.

**TABLE I. Major Functional Groups Available for Biocatalytic Modification***

A. **Hydroxyl Groups** -- These groups can undergo numerous reactions including oxidation to aldehydes or ketones (1.1), acylation with ester donors (2.3, 3.1), glycosidic bond formation (2.4, 3.2, 5.3), and etherification (2.1, 3.3). Potential for stereo- and regio-selective synthesis as well as prochiral specificity.

B. **Aldehydes and Ketones** -- These groups can undergo selective reduction to alcohols (1.1). This may then be followed by modifications of hydroxyl groups.

C. **Amino Groups** These groups can undergo oxidative deamination (1.4), N-dealkylation (1.5, 1.11), transferred to other compounds (2.6), peptide bond synthesis (3.4, 6,3), and acylation with ester donors (2.3, 3.1).

D. **Carboxyl Groups** -- These groups can be decarboxylated (1.2, 1.5, 4.1), and esterified (3.1, 3.6).

E. **Thiol Groups** -- These groups can undergo reactions similar to hydroxyls, such as thioester formation (2.8, 3.1), thiol oxidation (1.8), and disulfide formation (1.8).

F. **Aromatic Groups** -- These groups can hydroxylated (1.11, 1.13, 1.14), and oxidatively cleaved to diacids (1.14).

G. **Carbohydrate Groups** -- These groups can be transferred to hydroxyls and phenols (2.4, 3.2, 5.3), and to other carbohydrates (2.4).

H. **Ester and Peptide Groups** -- These groups can be hydrolyzed (3.1, 3.4, 3.5, 3.6, 3.9), and transesterified (or interesterified) (3.1, 3.4).

I. **Sulfate and Phosphate Groups** -- These groups can be hydrolyzed (3.1, 3.(, transferred to other compounds (2.7, 2.8), and esterified (3.1).

J. **Halogens** -- These groups can be oxidatively or hydrolytically removed (1.11, 3.8), and added (1.11).

K. **Aromatic Amines and Phenols** -- These groups can be acylated (2.3, 3.1) or oxidatively polymerized (1.10, 1.11, 1.14).

*Numbers in parentheses correspond to the EC (Enzyme Commission) categorization of enzymes and enzyme classes.

TABLE II. A Representative Subset of Biocatalytic Reactions Which May Be Used to Modify Functional Groups

1. Oxidation of primary and secondary alcohols; Reduction of aldehydes and ketones.

Reaction Boxes: A1, B1, C2, D2

Enzyme Class: 1.1. Dehydrogenases, Dehydtratases, Oxidases

Representative Enzymes:

Alcohol dehydrogenase
Glycerol Dehydrogenase
Glycerol-3-Phosphate Dehydrogenase
Xylulose Reductase
Polyol Dehydrogenase
Sorbitol Dehydrogenase
Glyoxylate Reductase
Lactate Dehydrogenase
Glycerate Dehydrogenase
ß-Hydroxybutyrate Dehydrogenase
Malate Dehydrogenase
Glucose Dehydrogenase
Glucose-6-Phosphate Dehydrogenase
$3\alpha$- and $3\beta$-Hydroxysteroid Dehydrogenase
$3\alpha$-, $20\beta$-Hydroxsteroid Dehydrogenase
Fucose Dehydrogenase
Cytochrome-Dependent Lactate Dehydrogenase
Galactose Oxidase
Glucose Oxidase
Cholesterol Oxidase
Alcohol Oxidase
Glycolate Oxidase
Xanthine Oxidase
Fructose Dehydrogenase

Cosubstrates/Cofactors: NAD(P)(H)

**TABLE II (cont.)**

2. Acylation of primary and secondary alcohols.

Reaction Boxes: A3, B4

Enzyme Classes: 3.1, 3.4, 3.5, 3.6

Representative Enzymes: Esterases, lipases, proteases, sulfatases, phosphatases, acylases, lactamases, nucleases, acyl transferases

Esterases
Lipases
Phospholipase A
Acetylesterase
Acetyl Cholinesterase
Butyryl Cholinesterase
Pectinesterase
Cholesterol Esterase
Glyoxalase II
Alkaline Phosphatase
Acid Phosphatase
A Variety of nucleases
Glucose-6-Phosphatase
Fructose 1,6-Diphosphatase
Ribonuclease
Deoxyribonuclease
Sulfatase
Chondro-4-Sulfatase
Chondro-6-Sulfatase
Leucine Aminopeptidase
Carboxypeptidase A
Carboxypeptidase B
Carboxypeptidase Y
Carboxypeptidase W
Prolidase
Cathepsin C
Chymotrypsin
Trypsin
Elastase

TABLE II (cont.)

| |
|---|
| Subtilisin |
| Papain |
| Pepsin |
| Ficin |
| Bromelain |
| Rennin |
| Proteinase A |
| Collagenase |
| Urokinase |
| Asparaginase |
| Glutaminase |
| Urease |
| Acylase I |
| Penicillinase |
| Cephalosporinase |
| Creatininase |
| Guanase |
| Adenosine Deaminase |
| Creatine Deaminase |
| Inorganic Pyrophosphatase |
| ATPase |
| Choline Acetyltransferase |
| Carnitine Acetyltransferase |
| Phosphotransacetylase |
| Chloramphenicol Acetyltransferase |
| Transglutaminase |
| γ-Glutamyl Transpeptidase |

TABLE II (cont.)

Cosubstrates/Cofactors: Esters of alkyl, aryl, charged, polar/neutral groups. These acyl donors can be chosen from the class consisting of the following structural formulae:

R-O-CO-R'

Where R = alkyl, vinyl, isopropenyl haloalkyl, aryl, derivatives of aryl (i.e., nitrophenyl) and R' can be any alkyl or aryl group with or without derivatives. Such derivatives include halogens, charged functional groups (i.e., acids, sulfates, phosphates, amines, etc.), glycols (protected or unprotected), etc.

3. Transglycosylation of primary and secondary alcohols.

Reaction Boxes: A10,B10

Enzyme Class: 2.4, 3.2

Representative Enzymes:

Phosphorylase a
Phosphorylase b
Dextransucrase
Levansucrase
Sucrose Phosphorylase
Glycogen Synthase
UDP-Glucuronyltransferase
Galactosyl Transferase
Nucleoside Phosphorylase
α- and β-Amylase
Amyloglucosidase (Glucoamylase)
Cellulase
Dextranase
Chitinase
Pectinase
Lysozyme
Neuraminidase
Xylanase
α- and ß-Glucosidase

TABLE II (cont.)

α- and ß-Galactosidase
α- and ß-Mannosidase
Invertase
Trahalase
ß-N-Acetylglucosaminidase
γ-Glucuronidase
Hyaluronidase
ß-Xylosidase
Hesperidinase
Pullulanase
α-Fucosidase
Agarase
Endoglycosidase F
NADase
Glycopeptidase F
Thioglucosidase

Cosubstrates/Cofactors: All available sugars and their derivatives. These sugars can be monosaccharides, disaccharides, and oligosaccharides and their derivatives.

4. Etherification of primary and secondary alcohols

Reaction Boxes: A11, B11

Enzyme Classes: 2.1, 3.2

Representative Enzymes:
Catechol α-Methyltransferase
Aspartate Transcarbamylase
Ornithine Transcarbamylase
S-Adenosylhomocysteine Hydrolase

Cosubstrates/Cofactors: Alcohols or ethers of any chain length.

TABLE II (cont.)

5. Acylation of primary and secondary amines.

Reaction Boxes: E3, E4
Enzyme Classes: 2.3, 3.1, 3.4, 3.5, 3.6

Representative Enzymes:

Choline Acetyltransferase
Carnitine Acetyltransferase
Phosphotransacetylase
Chloramphenicol Acetyltransferase
Transglutaminase
γ-Glutamyl Transpeptidase
Esterases
Lipases
Phospholipase A
Acetylesterase
Acetyl Cholinesterase
Butyryl Cholinesterase
Pectinesterase
Cholesterol Esterase
Glyoxylase II
Alkaline Phosphatase
Acid Phosphatse
A Variety of nucleases
Glucose-6-Phosphatase
Fructose 1,6-Diphosphatase
Ribonuclease
Deoxyribonuclease
Sulfatase
Chondro-4-Sulfatase
Chondro-6-Sulfatase
Leucine Aminopeptidase
Carboxypeptidase A
Carboxypeptidase B
Carboxypeptidase Y
Carboxypeptidase W

**TABLE II (cont.)**

Prolidase
Cathepsin C
Chymotrypsin
Trypsin
Elastase
Subtilisin
Papain
Pepsin
Ficin
Bromelin
Rennin
Proteinase A
Collagenase
Urokinase
Asparaginase
Glutaminase
Urease
Acylase I
Pinicillinase
Cephalosporinase
Creatininase
Guanase
Adenosine Deaminase
Creatine Deaminase
Inorganic Pyrophosphatase
ATPase

Cosubstrates/Cofactors: See example number 2, above.

6. Esterification of carboxylic acids.

Reaction Boxes: 17.

Enzyme Classes: 3.1, 3.6

TABLE II (cont.)

Representative Enzymes:

- Esterases
- Lipases
- Phospholipase A
- Acetylesterase
- Acetyl Cholinesterase
- Butyryl Cholinesterase
- Pectinesterase
- Cholesterol Esterase
- Glyoxylase II
- Alkaline Phosphatase
- Acid Phosphatase
- A Variety of nucleases
- Glucose-6-Phosphatase
- Fructose 1,6-Diphosphatase
- Ribonuclease
- Deoxyribonuclease
- Sulfatase
- Chondro-4-Sulfatase
- Chondro-6-Sulfatase
- Inorganic Pyrophosphatase
- APTase

Cosubstrates/Cofactors: alcohols of any chain length being alkyl, aryl, or their structural derivatives.

**TABLE III.** **Biocatalytic "Reaction Box" Matrix Approach**

| | | A Primary Hydroxyl | B Secondary Hydroxyl | C Ketone | D Aldehyde | E Primary Amine | F Secondary Amine | G Tertiary Amine | H Thiol | I Carboxylic Acid | J Ester |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Reaction Type | | 1-OH | 2-OH | $R_2C=O$ | R-CHO | $1\text{-}NH_2$ | 2-NHR | 3-NR2 | SH(orR) | COOH | COOR |
| Oxidation | 1 | 1 | 1 | | 1 | | | | | | |
| Reduction | 2 | | | 1 | 1 | | | | | | 2 |
| Acylation-Primary | 3 | >30 | | | | >30 | | | >30 | | |
| Acylation-Secondary | 4 | | >30 | | | >30 | | | >30 | | |
| Transesterification | 5 | | | | | | | | | | >30 |
| Interesterification | 6 | | | | | | >30 | >30 | | | >30 |
| Esterification | 7 | | | | | | | | | >100 | |
| Hydrolysis | 8 | | | | | | | | | | 1 |
| Peptide Formation | 9 | | | | | | | | | >100 | |
| Transglycosylation | 10 | >30 | >30 | | | >30 | >30 | >30 | >30 | | |
| Etherification | 11 | >30 | >30 | | | | | | | | |

**TABLE III (cont. 1)**

**Biocatalytic "Reaction Box" Matrix Approach**

| | A | B | C | D | E | F | G | H | I | J |
|---|---|---|---|---|---|---|---|---|---|---|
| Dealkylation 12 | | | | | | 1 | 2 | 1 | | |
| Hydroxylation 13 | | | | | | | | | | |
| Deamination 14 | | | | | 1 | | | | | |
| Ring Cleavage 15 | | | | | | | | | | |
| Isomerization 16 | | | | | | | | | | |
| Ligation 17 | | | | | | | | | >100 | |
| Oxidative Polymerization 18 | | | | | | | | | | |
| Hydration/Amination 19 | | | | | | | | | | |
| Decarboxylation 20 | | | | | | | | | 1 | |
| Transaldoases/trans ketolases 21 | | | >30 | >30 | | | | | | |
| Dehalogenation 22 | | | | | | | | | | |

**TABLE III (cont. 2)**

**Biocatalytic "Reaction Box" Matrix Approach**

| | | K Carbo-hydrate | L Sulfate | M Phos-phate | N Halogen | O Aromatic Amines | P Phenols | Q Aromatic groups | R Double Bonds | S Amides/ Peptides |
|---|---|---|---|---|---|---|---|---|---|---|
| Reaction Type | | Carbohy | $SO_4$ | $PO_4$ | C-X | Ph-$NR_2$ | Ph-OH | $C_6H_5$ | C=C | CONR |
| Oxidation | 1 | 24 | | | | | | | | |
| Reduction | 2 | 1 | 3 | | | | | | | |
| Acylation-Primary | 3 | >30 | | | | | | | | |
| Acylation-Secondary | 4 | >30x4 | | | | >30 | >30 | | | |
| Transesterification | 5 | | >30 | >30 | | | | | | |
| Interesterification | 6 | | | | | | | | | >30 |
| Esterification | 7 | | >100 | >100 | | | | | | |
| Hydrolysis | 8 | | 1 | 1 | | | | | | 1 |
| Peptide Formation | 9 | | | | | | | | | |
| Transglycosylation | 10 | >30 | | | | | >30 | | | |
| Etherification | 11 | >30 | | | | | | | | |

**TABLE III (cont. 3)**

**Biocatalytic "Reaction Box" Matrix Approach**

| | K | L | M | N | O | P | Q | R | S |
|---|---|---|---|---|---|---|---|---|---|
| Dealkylation 12 | | | | | 2 | | | | |
| Hydroxylation 13 | | | | | | >3 | 5 | | |
| Deamination 14 | | | | | 1 | | | | |
| Ring Cleavage 15 | | | | | | 3 | 4 | | |
| Isomerization 16 | | | | | | | | | |
| Ligation 17 | | | | | | | | | |
| Oxidative Polymerization 18 | | | | | 3 | 3 | | | |
| Hydration/Amination 19 | | | | | | | | 2 | |
| Decarboxylation 20 | | | | | | | | | |
| Transaldolase/Transketolase 21 | | | | | | | | | |
| Dehalogenation 22 | | | | 1 | | | | | |

**TABLE IV. Reaction Box Analysis of AZT Derivatization Indicating the Total Number of Possible Reaction Products**

| Reaction Box | Number of Possible Products |
|---|---|
| A3 | 30[a] |
| A10 | 30[b] |
| A11 | 30 |
| C2 | 2 x 30[c] |
| C2 | 2 x 30[c] |
| G6 | 30 |
| G10 | 30 |
| G12 | x2 |
| Total | $1.75 \times 10^{11}$ distinct compounds[d] |

(a) Assuming 30 different acyl donors to be added to this reaction mixture. This includes alkyl, aryl, and of different lengths, (b) Assuming 30 UDP-sugars used in this reactin box, (c) Reduction of the ketones to secondary alcohols leads to the potential acylation of the secondary alcohols and adds 30-fold more potential products; and (d) Each box's possible permutations are multiplied together to estimate the total number of compounds synthesized.

**TABLE V. Reaction Box Analysis of Established Drugs Indicating the Total Number of Possible Reaction Products**

| Starting Compound | Number of Functional Groups | Estimated Number of Derivatives |
|---|---|---|
| Castanospermine | 4 | 810,000 |
| Cyclosporin | 24 | billions |
| Gentamicin | 8 | billions |
| Haloperidol | 3 | 120 |
| Methotrexate | 7 | greater than $10^{19}$ |
| Muscarine | 2 | 2,400 |
| Prazosin | 6 | 288,000 |
| Prednisone | 12 | 46,080,000 |
| Thyroxine | 3 | 2,160,000 |
| Valproic Acid | 1 | 900 |
| Vancomycin | many | billion |

**TABLE VI. Representative Subset of Screening Assays Possible to Test for Anti-Cancer, Anti-Viral and Anti-Hypertensive Activities**

**Anti-Cancer Drugs**

1. KB (Eagle) cell culture assay
2. Inhibition of the growth of human breast cancer cell lines *in vitro*
3. Inhibition of the growth of P388 leukemia cells *in vitro*
4. Inhibition of the growth of murine L1210 cells *in vitro*
5. Inhibition of gylcinamide ribonucleotide formyltransferase activity
6. Inhibition of ribonucleotide reductase activity
7. Inhibition of protein kinase C activity
8. Inhibition of human aromatase activity
9. Inhibition of DNA topoisomerase II activity
10. Inhibition of dihydrofolate reductase
11. Inhibition of aminoimidazole carboxamide ribonucleotide formyltransferase

**Anti-AIDS Drugs**

1. Inhibition of HIV virus replication devoid of cytotoxic activity
2. Inhibition of HIV protease activity
3. Soluble-formazan assay for HIV-1
4. Inhibition of HIV reverse transcriptase activity

**Anti-Hypertensive Drugs**

1. Inhibition of ACE activity
2. Inhibition of human plasma renin
3. Inhibition of in vitro human renin
4. Inhibition of angiotensin converting enzyme
5. Alpha 1-adrenergic receptor binding assay
6. Alpha 2-adrenergic receptor binding assay
7. Beta-adrenergic receptor binding assay (bronchodilator, cardiotonic, tocolytic, anti-anginal, anti-arrhythmic, anti-glaucoma)
8. Dopamine receptor binding assay (anti-migraine, anti-parkinsonian, anti-emetic, anti-psychotic)

## Example 1

1. Enzymatic esterification of taxol

1.1 Analysis of reaction products

Products of enzymatic esterification of taxol were analyzed using reversed-phase HPLC on a Waters system with a 990 photodiode array detector, and a 3.9 x 300 mm μBondapak $C_{18}$ column. The solvent system used consisted of a water:acetonitrile mixture (40:15 *v/v*) and isopropanol. Elution was performed at 1 ml/min according to the following linear gradient program:

| Time (min) | Solvent Composition (*v/v* %) | |
|---|---|---|
| | Water/acetonitrile | Isopropanol |
| 0-8 | 72 | 28 |
| 8-16 | 50 | 50 |
| 16-24 | 0 | 100 |
| 26-28 | 0 | 100 |
| 28-30 | 72 | 28 |

Product peaks were detected at 227 nm using a photodiode array detector.

1.2 Enzymatic synthesis of taxol esters - Identification of active biocatalysts (Reaction Box B-4)

A mixture of 55 enzymes (Table VII) consisting of crude lipases, crude proteases, and purified proteases (total mass of 600 mg) was added to a solution of taxol (17.0 mg, 20 μmol) and vinyl butyrate (0.25 ml, 1.5 mmol) in 2.9 ml tert amyl alcohol. The mixture was placed into a septum-sealed glass vial, sonicated for 30 s, and put into an orbit shaker operating at 250 rpm and 35°C for a period of 48 h. The reaction was then stopped by removing the suspended solid catalyst by centrifugation. The supernatant containing taxol esters was evaporated to dryness in vacuum and redissolved in 0.3 ml methanol. An aliquot of the resultant concentrated solution (15 μl) was diluted with 250 μl methanol and analyzed by HPLC as described in 1.1. The chromatogram revealed a peak with a retention time of 9.7 min (unreacted taxol), and a peak at 14.6 min (2'-taxol butyrate, yield 7%).

The enzymes were then split into three groups: 26 lipases (100 mg/ml), 26 crude proteases (100 mg/ml), and 4 purified proteases (10 mg/ml) and the identical reaction as above performed. Only the purified proteases showed significant activity with a yield of 2'-butyrate of 15% after 48 h. The purified proteases were then divided into individual enzymes (all used at a concentration of 10 mg/ml) and thermolysin (a bacterial protease from *Bacillus thermoproteolyticus rokko a.k.a.* thermolysin) was identified as the most active enzyme with a yield after 48 h of 24%. This approach demonstrates that active biocatalysts can be easily identified by a sequential process of eliminating unreactive biocatalysts.

TABLE VII. Enzymes used in the acylation of taxol

## I. Lipases and Esterases

| Enzyme | Source | Company |
|---|---|---|
| CES | | Amano |
| L-10 | | Amano |
| G | *Penicillium sp.* | Amano |
| N | *Rhizopus niveus* | Amano |
| AP | *Aspergillus niger* (acid stable) | Sigma |
| Wheat Germ | | Amano |
| GC-20 | *Geotrichwn candidum* | Amano |
| AY-30 | *Candida rugosa* | Amano |
| P | *Pseudomonas cepacia* | Amano |
| AG-975 | | Sigma |
| Porcine Pancreatic | | Amano |
| APF | *A. niger* | Amano |
| R-10 | | Amano |
| AK | *Pseudomonas sp.* | Amano |
| PGE | Calf tongue root | Amano |
| D | | Amano |
| GC-4 | | Amano |
| CE | | Sigma |
| *Candida rugosa* | | Amano |
| FAP-15 | *Rhizopus sp.* | Amano |
| MAP-10 | *Mucor sp.* | Amano |
| Enzeco K16825 | | Enzeco |
| Lamb Pre-Gastric Esterase | | Quest |
| Calf Pre-Gastric Esterase | | Quest |
| Esterase 30,000 #3586 | | Gist Brocades |
| Lipase 80,000 #5093 | *Rhizopus sp.* | Gist Brocades |

TABLE VII. (cont.) Enzymes used in the acylation of taxol

## II. Proteases

| Enzyme | Source | Company |
|---|---|---|
| B | *Penicillium sp.* | Amano |
| Papain | Papaya | Sigma |
| 2A | *Aspergillus oryzae* | Amano |
| Proleather | *Bacillus sp.* | Amano |
| N | *B. subtilis* (neutral) | Amano |
| Acid Stable | *Rhizopus sp.* | Amano |
| Alcalase-2T | *B. Licheniformis* (Subtilisin Carlsberg) | Novo |
| Bromelain | Pineapple | Sigma |
| Alkaline Protease | | Quest |
| Fungal Protease #9240810 | | Biocon |
| Acid Protease #8221108 | | Biocon |
| Neutral Protease 900,000 #6W16B | | Biocon |
| HT | | MKC |
| Rapidase S-90 | *B. subtilis* | Gist Brocades |
| HT-Proteolytic 200 | | Solvay |
| Opticlean M-375 | Alkaline protease (from *Bacillus sp*). | Solvay |
| Optimase M-440 | Alkaline protease (from *Bacillus sp*). | Solvay |
| Fungal Protease 60,000 | | Solvay |
| Esperase-4T | Alkaline protease (from *Bacillus sp*). | Novo |
| Peptidase A | *A. oryzae* | Amano |
| Prozyme 6 | *Aspergillus sp.* | Amano |
| Protease M | *A. oryzae* | Amano |
| Newlase A | *A. niger* (acid stable) | Amano |
| Acylase Conc. | *Aspergillus sp.* | Amano |
| Protease S | *Bacillus sp.* (thermostable) | Amano |
| Acylase I | Porcine kidney | Sigma |

## III. Purified Enzymes

| Enzyme | Source | Company |
|---|---|---|
| Trypsin | Bovine pancreas | Sigma |
| Chymotrypsin | Bovine pancreas | Sigma |
| Subtilisin Carlsberg | *Bacillus liquefaciens* | Sigma |
| Protease X | *B. thermoproteolyticus rokko* | Sigma |

1.3. Enzymatic synthesis of a mixture of taxol esters - Sequential reaction box derivatization of taxol

The optimal enzyme catalyst used for the synthesis was produced by freeze-drying an aqueous solution containing thermolysin, KCl and potassium phosphate buffer adjusted to pH 7.5. The solid catalyst obtained after free-drying contained 5% enzyme, 94% KCl and 1% potassium phosphate. The powdered enzyme catalyst (335 mg - containing 16.75 mg thermolysin) was added to a solution of taxol (17.0 mg, 20 µmol) and vinyl caproate (straight-chain $C_6$ ester) (0.24 ml. 1.5 mmol) in 2.9 ml tert-amyl alcohol. The mixture was placed into a septum-sealed glass vial, sonicated for 30 s, and put into an orbit shaker operating at 250 rpm and 35°C. After 28 h the reaction mixture (including enzyme) was removed and the full contents of the mixture added to a separate solution containing vinyl propionate (0.24 ml, 2.2 mmol), vinyl acrylate (0.24 ml, 2.2 mmol), and vinyl butyrate (0.24 ml, 1.9 mmol). This second reaction was placed on the shaker and incubated at 250 rpm at 35°C. After 24 h, the reaction mixture (including enzyme) was removed and the full contents of the mixture added to a separate solution containing vinyl acetate (0.25 ml, 2.6 mmol) and vinyl chloroacetate (0.24 ml, 1.8 mmol). This reaction was allowed to proceed for 24 h at 250 rpm and 35°C. The sequential reaction was then stopped by removing the suspended solid catalyst by centrifugation. The sequential reaction was aided by the soluble nature of the taxol and taxol derivatives. The supernatant containing taxol esters was evaporated to dryness in vacuum and redissolved in 0.3 ml methanol. An aliquot of the resultant concentrated solution (15 µl) was diluted with 250 µl methanol and analyzed by HPLC as described in 1.1. The chromatogram revealed a peak with retention time 9.7 min (unreacted taxol), a broad peak between 11 and 13 min (total estimated yield 52%), peaks at 14.6 min (yield 9%) and 17.4 min (yield 18%), and a small peak at 18.4 min (yield 0.2%). Total reaction yield was approximately 80%.

1.4 Removal of taxol from reaction mixture

A concentrated methanol solution of reaction products produced as described in 1.3 was applied on a preparative TLC silica plate (Whatman, 20x20 cm, silica layer thickness 500 µm, containing fluorescent marker), and the plates were developed using a solvent mixture of chloroform:acetonitrile (4:1 *v/v*). Positions of product spots were determined by irradiating the plates with ultraviolet light. The $R_f$ value of taxol is 0.16 and the $R_f$ of the taxol esters range from 0.28 to 0.71. The products were removed from the TLC plate and dissolved in ethyl acetate. The products were then dried *in vacuo*.

1.5 Screening the mixture of taxol esters

The library of taxol derivatives described above was screened for cytotoxicity against HL-60 cells, a promyelocytic leukemia cell line, and MOLT-4 cells, a lymphoblastic leukemia cell line. Cells were seeded in 96-well plates at densities of 30,000 cells/well and grown in RPMI-1640 medium containing 10% bovine fetal calf serum at 37°C for 24 h. The medium was then replaced

with fresh medium containing the taxol derivatives (excluding taxol, which had been removed by preparative thin-layer chromatography) dissolved in DMSO at final concentrations ranging from 100 nM to 0.1 nM. The final concentration of DMSO in the cell medium was 0.5% (*v/v*). After 72 h, samples were removed for cell counts. Total cell number and viability were determined by trypan blue exclusion and manual cell counting on a hemacytometer. The dose-response data are reported in Table IX. The hemacytometer cell counts revealed that the taxol-derivative library contained at least one cytotoxic derivative.

1.6 Backtracking to identify active product(s)

1.6.1 Enzymatic synthesis of individual taxol esters - Identification of active taxol esters by building-up the reactions comprising taxol acylation in 1.3 above.

Given the predictable nature of the enzymatic acylation reactions, it is possible to identify the possible products without analyzing the chemical compositions of the mixture. The possible products were, therefore, synthesized individually, as described below, and screens on these individual ester products were then initiated as part of the backtracking process.

The powdered enzyme catalyst prepared as described in 1.3 above (140 mg) was added to a solution of taxol (5.5 mg, 6.5 μmol) and on individual vinyl ester (80 μl, approximately 2 mmol) in 1.0 ml tert-amyl alcohol. The following vinyl esters were used as acylating agents: acetate, chloroacetate, acrylate, propionate, butyrate, and caproate. Each mixture was placed into a septum-sealed glass vial, sonicated for 30 s, and put into an orbit shaker operating at 250 rpm and 35°C. After 96 h the reaction was stopped by removing the suspended solid catalyst by centrifugation. Supernatants containing taxol esters were evaporated to dryness *in vacuo* and redissolved in 0.3 ml methanol each. An aliquot of each resultant concentrated solutions (20 μl) was diluted with 80 μl methanol and analyzed by HPLC as described in 1.1. Results of HPLC analysis are given in Table VIII.

**Table VIII. Retention times (RT) and $R_f$ values of Taxol esterification products***

| Ester | 2'-ester | | | 7-ester | | | 2',7-diester | | | Total yield (%) |
|---|---|---|---|---|---|---|---|---|---|---|
| | RT (min) | $R_f$ | Yield (%) | RT (min) | $R_f$ | Yield (%) | RT (min) | $R_f$ | Yield (%) | |
| Acetate | 10.5 | 0.28 | 57 | 13.1 | 0.48 | 30 | 15.2 | 0.57 | 2 | 89 |
| Acrylate | 12.2 | 0.39 | 80 | 14.1 | 0.56 | 10 | 15.6 | 0.67 | 0.4 | 90 |
| Chloroacetate | 12.5 | 0.32 | 70 | 15.9 | 0.51 | 3 | 0.71 | 0.71 | 12 | 85 |
| Propionate | 12.8 | 0.39 | 78 | 14.5 | 0.58 | 12 | – | – | – | 90 |
| Butyrate | 14.5 | 0.41 | 67 | 15.8 | 0.61 | 11 | – | – | – | 78 |
| Hexanoate | 17.3 | 0.47 | 50 | 18.1 | 0.67 | 7 | – | – | – | 57 |

*Taxol: retention time 9.7 min. $R_f$ 0.16.

1.6.2. Recovery of reaction products by thin layer chromatography (TLC)

Individual ester reaction products were separated from taxol by TLC. Concentrated methanol solutions of reaction products produced as described in 1.6 were applied on preparative TLC silica plates (Whatman, 20x20 cm, silica layer thickness 500 μm, containing fluorescent market), and the plates were developed using a solvent mixture of chloroform:acetonitrile (4:1 *v/v*). Positions of product spots were determined by irradiating the plates with ultraviolet light. The $R_f$ values of the products are given in Table VIII. Product spots were scraped off the plates separately and scrapings were eluted with 15 ml ethyl acetate to recover the product. Dry products were obtained by evaporation of ethyl acetate *in vacuo*.

1.6.3 Screening individual taxol esters

To determine which of the newly synthesized product(s) was active, the synthetic history of the library was "backtracked" by enzymatically synthesizing all possible ester products individually (as described above). Following isolation of the products by TLC, each derivative was tested for cytotoxic activity as described above. The cytotoxicity experiments revealed that two derivatives, 2'-chloroacetyltaxol and 2'-acryloyltaxol, are active against both cell lines (Table IX).

**Table IX. Percent Viability of MOLT-4 and HL-60 Cells**
After 72 h exposure to taxol and its derivatives at various concentrations

| | 100 nM | 10 nM | 1 nM | 0.1 nM |
|---|---|---|---|---|
| **MOLT-4** | | | | |
| Taxol | 1.22 | 11.1 | 92.8 | 92.9 |
| Library | 3.13 | 67.9 | 95.9 | 95.0 |
| 2'-Chloroacetyltaxol | 4.26 | 91.9 | 94.4 | 96.9 |
| 2'-Acryloyltaxol | 2.70 | 61.0 | 95.6 | 91.0 |
| **HL-60** | | | | |
| Taxol | 0.00 | 32.6 | 96.1 | 98.3 |
| Library | 5.26 | 98.3 | 98.5 | 99.0 |
| 2'-Chloroacetyltaxol | 2.13 | 99.5 | 97.3 | 99.5 |
| 2'-Acryloyltaxol | 4.69 | 87.1 | 96.9 | 97.5 |

**Example 2**

2. Enzymatic hydrolysis of taxol

2.1 Analysis of reaction products

Products of enzymatic hydrolysis of taxol were analyzed using reversed-phase HPLC on a Waters system with a 990 photodiode array detector, with 3.9 x 300 mm µBondapak $C_{18}$ column. The solvent system used consisted of water and acetonitrile. The solvents were fed into the HPLC system at 1 ml/min according to the following linear gradient program:

| Time (min) | Solvent Composition (*v/v %*) | |
|---|---|---|
| | Acetonitrile | Water |
| 0-1 | 25 | 75 |
| 1-5 | 45 | 55 |
| 5-26 | 58 | 42 |
| 26-30 | 100 | 0 |
| 30-35 | 25 | 75 |

Product peaks were detected at 227 nm using a photodiode array detector.

2.2 Enzymatic hydrolysis of taxol (Reaction Boxes J-8 and S-8)

The enzyme catalyst used for hydrolysis was produced by freeze-drying an aqueous solution (adjusted to pH 7.5) containing equal weight amounts of thermolysin, subtilisin Carlsberg, chymotrypsin and trypsin. The enzyme catalyst (1.1 mg) was dissolved in 0.7 ml 0.1 M potassium phosphate buffer pH 7.5. The aqueous enzyme solution was added to a solution of taxol (2.0 mg, 2.4 µmol) in 0.3 ml tert amyl alcohol. The biphasic reaction system produced in

this way was placed into a septum-sealed glass vial and put on an orbit shaker operating at 75 strokes/min and 20°C. After 26 h the organic solvent layer was separated, evaporated to dryness and redissolved in 0.2 ml methanol for HPLC analysis. The aqueous phase was diluted with 3.5 ml methanol and insoluble solids (precipitated enzymes) were removed by centrifugation. Clear supernatant was evaporated into dryness and redissolved in 0.2 ml methanol for HPLC analysis. HPLC analysis was performed as described in 2.1. Neither taxol, nor any hydrolysis products were detected in the methanol solution obtained after workup of the aqueous phase of the biphasic reaction system. On the other hand, the chromatogram of the methanol solution of the dry residue obtained from the organic layer revealed a peak with retention time of 20.4 min (unreacted taxol) and a peak at 18.4 min, representing a product of enzymatic hydrolysis of taxol, as identified by the characteristic uv-absorbance scan. The estimated yield of this product was 9%.

## Example 3

3. Enzymatic glycosylation of taxol

3.1 Analysis of Reaction Products

Products of enzymatic glycosylation of taxol were analyzed using reversed-phase HPLC on a Waters system with a 990 photodiode detector, with a 3.9 x 300 mm µBondapak $C_{18}$ column. The solvent system used consisted of water and methanol. The solvents were fed into the HPLC system at 1 ml/min according to the following linear gradient program:

| Time (min) | Solvent Composition (*v/v* %) | |
|---|---|---|
| | Methanol | Water |
| 0-5 | 25 | 75 |
| 5-37 | 85 | 15 |
| 37-40 | 100 | 0 |
| 40-42 | 100 | 0 |
| 42-44 | 25 | 75 |

Product peaks were detected at 227 nm using a photodiode array detector.

3.2 Enzymatic glycosylation of taxol

Glycosylation of taxol was performed using α-glucosidase from baker's yeast as a biocatalyst. For reference, the enzymes used were: α-glucosidase from brewer's yeast (pH 7.0), α-glucosidase from baker's yeast (pH 7.0), β-galactosidase from *E. coli* (pH 5.0), β-galactosidase from *A. oryzae* (pH 5.0), β-glucuronidase from bovine liver (pH 5.0). These enzymes were used in two mixtures (one at pH 7.0 and one at pH 5.0) and it was determined that only the mixture at pH 7.0 was active in glycosylating taxol. This mixture was then divided into individual enzymes where it was found that α-glucosidase from baker's yeast was the active biocatalyst.

WHAT IS CLAIMED IS:

1. A method for drug identification comprising:
   (a) conducting a series of biocatalytic reactions by mixing biocatalysts with a starting compound to produce a reaction mixture and thereafter a library of modified starting compounds;
   (b) testing the library of modified starting compounds to determine if a modified starting compound is present within the library which exhibits a desired activity;
   (c) identifying the specific biocatalytic reactions which produce the modified starting compound of desired activity by systematically eliminating each of the biocatalytic reactions used to produce a portion of the library, and then testing the compounds produced in the portion of the library for the presence or absence of the modified starting compound with the desired activity; and
   (d) repeating the specific biocatalytic reactions which produce the modified compound of desired activity and determining the chemical composition of the reaction product.

2. A method for drug identification of Claim 1 wherein
   (a) the biocatalytic reactions are conducted with a group of biocatalysts that react with distinct structural moieties found within the structure of a starting compound,
   (b) each biocatalyst is specific for one structural moiety or a group of related structural moieties; and
   (c) each biocatalyst reacts with many different starting compounds which contain the distinct structural moiety.

3. A method for drug identification comprising:
   (a) conducting a series of biocatalytic reactions on a starting compound to produce a library of modified starting compounds;
   (b) providing a high affinity receptor for a starting compound of desired activity and exposing the modified compounds formed after each biocatalytic reaction to the high affinity receptor;
   (c) isolating the high affinity receptor-modified starting compound complex from the biocatalytic reaction mixture;
   (d) dissociating the complex into its component parts; and
   (e) determining the chemical composition of the dissociated modified starting compound.

4. A method for drug identification comprising:
   (a) adding a high affinity receptor to a starting compound to form a biocatalytic reaction mixture at a concentration that allows for essentially all of the high affinity receptor to bind with the starting compound and about an equal molar amount of starting compound remaining free in solution and available for biocatalytic modification;
   (b) producing a modified starting compound exhibiting a higher binding affinity than the starting compound resulting in the displacement of the starting compound from the high affinity receptor;
   (c) forming a complex of the modified starting compound and the high affinity receptor, thereby protecting the modified starting compound from further biocatalytic reaction;
   (d) isolating the complex from the biocatalytic reaction mixture;
   (e) dissociating the complex into its component parts;
   (f) determining the chemical composition of the dissociated modified starting compound; and
   (g) repeating steps (a) through (b) to produce a library of modified starting compounds.

5. A method for drug identification according to Claim 4 wherein the high affinity receptor is present on the surface or contained within a living cell or immobilized to a natural or artificial support.

6. A method for drug identification according to Claim 1 wherein the biocatalytic reactions are selected from a group consisting of:
   (a) Oxidation of primary and secondary alcohols;
   (b) Reduction of aldehydes and ketones;
   (c) Acylation of primary and secondary alcohols;
   (d) Transglycosylation of primary and secondary alcohols;
   (e) Etherification of primary and secondary alcohols;
   (f) Acylation of primary and secondary amines; and
   (g) Esterification of carboxylic acids.

7. A method for drug identification according to Claim 1 wherein the biocatalyst comprises crude or purified enzymes, cellular lysate preparations, partially purified lysate preparations, living cells and intact non-living cells, used in a soluble, suspended or immobilized form.

8. A method for drug identification according to Claim 1 wherein the biocatalytic reactions are used in combination with non-specific chemical reactions to produce a library of modified starting compounds.

9. A method for drug identification according to Claim 1 further comprising:
   (a) exposing the reaction mixture to a drug screening device that measures the binding of compounds with desired activity to localized or immobilized receptor molecules or cells;
   (b) correlating a positive measurement from the drug screening device with the sequence of biocatalytic reactions used to synthesize the reaction mixture and the specific reaction sequence producing the modified starting compound with desired activity; and
   (c) repeating the biocatalytic reaction sequence to produce the modified starting compound of desired activity and to determine its chemical composition.

10. A method for drug identification according to Claim 1 wherein the biocatalytic reaction is performed with a biocatalyst immobilized to magnetic particles forming a magnetic biocatalyst, the method further comprising
   (a) initiating the biocatalytic reaction by combining the immobilized biocatalyst with substrate(s), cofactor(s) and solvent/buffer conditions used for a specific biocatalytic reaction;
   (b) removing the magnetic biocatalyst from the biocatalytic reaction mixture to terminate the biocatalytic reaction, which is accomplished by applying an external magnetic field causing the magnetic particles with the immobilized biocatalyst to be attracted to and concentrate at the source of the magnetic field, thus effectively separating the magnetic biocatalyst from the bulk of the biocatalytic reaction mixture and allowing for the transferral of the reaction mixture minus the magnetic biocatalyst from a first reaction vessel to a second reaction vessel, leaving the magnetic biocatalyst in the first reaction vessel;
   (c) conducting a second biocatalytic reaction, completely independent of the first biocatalytic reaction, by combining a second biocatalyst immobilized to magnetic particles with the second reaction vessel containing the biocatalytic reaction mixture transferred from the first reaction vessel; and
   (d) repeating steps a) through c) to accomplish a sequential series of distinct and independent biocatalytic reactions and produce a corresponding series of modified starting compounds.

11. A method for drug identification according to Claim 10 wherein the biocatalytic reaction is performed with a biocatalyst immobilized to a particle and the biocatalyst is removed from the biocatalytic reaction mixture by centrifugation or filtration.

12. A method for drug identification according to Claim 1 using an automated robotic device, the automated robotic device comprised of:

(a) an XY table with an attached XYZ pipetting boom to add volumetric amounts of enzyme, substrate, cofactor, solvent solutions and assay reagents from reagent vessels positioned on the XY table to reaction and assay vessels positioned on the same XY table;

(b) an XYZ reaction-vessel transfer boom attached to the same XY table used to transfer reaction and assay vessels positioned on the XY table to different locations on the XY table;

(c) a temperature incubation block attached to the same XY table to house the reaction and assay vessels during reaction incubations and control the temperature of the reaction mixtures;

(d) a magnetic separation block attached to the same XY table to separate the biocatalyst immobilized to magnetic particles from the biocatalytic mixture by applying an external magnetic field causing the magnetic particles to be attracted to and concentrate at the source of the magnetic filed, thus effectively separating them from the bulk of the biocatalytic reaction mixture; and

(e) a programmable microprocessor interfaced to the XYZ pipetting boom, and XYZ reaction vessel transfer boom, the temperature block and the magnetic separation block to precisely control and regulate all movements and operations of these functional units in performing biocatalytic reactions to produce modified starting compounds and assays to determined desired activities.

13. A method for rapid drug development comprising:

(a) mixing a starting drug compound with a high affinity receptor at approximately one half the molar concentration of the starting drug compound to allow essentially all of the high affinity receptor to bind to the starting drug compound and leave an equal molar amount of starting drug compound free in solution and available for biocatalytic modification;

(b) producing a modified drug compound from the biocatalytic reaction between the starting drug compound and the receptor, the modified drug

compound having a higher binding affinity than the starting drug compound;

(c) displacing the starting drug compound from the receptor with the modified drug compound;

(d) protecting the bound modified drug compound from further biocatalytic reactions;

(e) dissociating the complex of the modified drug compound and receptor into its component parts;

(f) determining the chemical composition of the dissociated modified starting drug compound; and

(g) repeating steps a) through e) and thus conduct a series of biocatalytic reactions on a starting drug compound to produce a library of modified drug compounds with higher binding affinities.

14. A method for rapid drug development according to Claim 1 wherein the high affinity receptor is present on the surface or contained within a living cell or immobilized to a natural or artificial support.

15. A method for rapid drug development according to Claim 1 wherein the biocatalytic reactions are selected from a group consisting of those listed in Table II.

16. A method for rapid drug development according to claim 1 wherein the testing of the library of modified starting compounds is selected from a group of assays consisting of those listed in Table VI.

17. A method for rapid drug development according to claim 1 wherein the biocatalytic reaction is performed with a biocatalyst immobilized to magnetic particles forming a magnetic biocatalyst whereas:

(a) the biocatalytic reaction is initiated by combining the immobilized biocatalyst with the following to form the biocatalytic reaction mixture: substrate(s), cofactor(s) and solvent/buffer conditions used for the specific biocatalytic reaction;

(b) the biocatalytic reaction is terminated by removing the magnetic biocatalyst from the biocatalytic reaction mixture, which is accomplished by applying an external magnetic field causing the magnetic particles with the immobilized biocatalyst to be attracted to and concentrate at the source of the magnetic field, thus effectively separating the magnetic biocatalyst from the bulk of the biocatalytic reaction mixture and allowing for the removal of

the reaction mixture minus the magnetic biocatalyst to a second reaction vessel, leaving the magnetic biocatalyst in the first reaction vessel;

(c) a second biocatalytic reaction, completely independent of the first biocatalytic reaction, is initiated by adding a second biocatalyst immobilized to magnetic particles to the second reaction vessel containing the biocatalytic reaction mixture from the first biocatalytic reaction minus the magnetic biocatalyst used in the first biocatalytic reaction;

(d) the above described biocatalytic reaction cycle is repeated thus accomplishing a series of distinct and independent biocatalytic reactions producing a corresponding series of modified drug compounds.

(e) a magnetic separation block attached to the same XY table as above to separate the biocatalyst immobilized to magnetic particles from the biocatalytic reaction mixture by applying an external magnetic field causing the magnetic particles to be attracted to and concentrate at the source of the magnetic field, thus effectively separating them from the bulk of the biocatalytic reaction mixture; and

(f) a programmable microprocessor interfaced to the XYZ pipetting boom, the XYZ reaction vessel transfer boom, the temperature block and the magnetic separation block to precisely control and regulate all movements and operations of these functional units in performing biocatalytic reactions to produce modified starting compounds and assays to determined desired activities.

18. A method according to Claims 1, 3, 4 and 13 wherein the starting compounds are soluble in the reaction mixture.

19. A method according to Claim 18 wherein the starting compounds and resulting derivatives are soluble, and remain soluble for use in screening procedures.

20. A method according to Claims 1 wherein identifying the specific biocatalytic reactions which produce the modified starting compound of desired activity is done by systematically building up each of the biocatalytic reactions used to produce a portion of the library.

FIG. 1

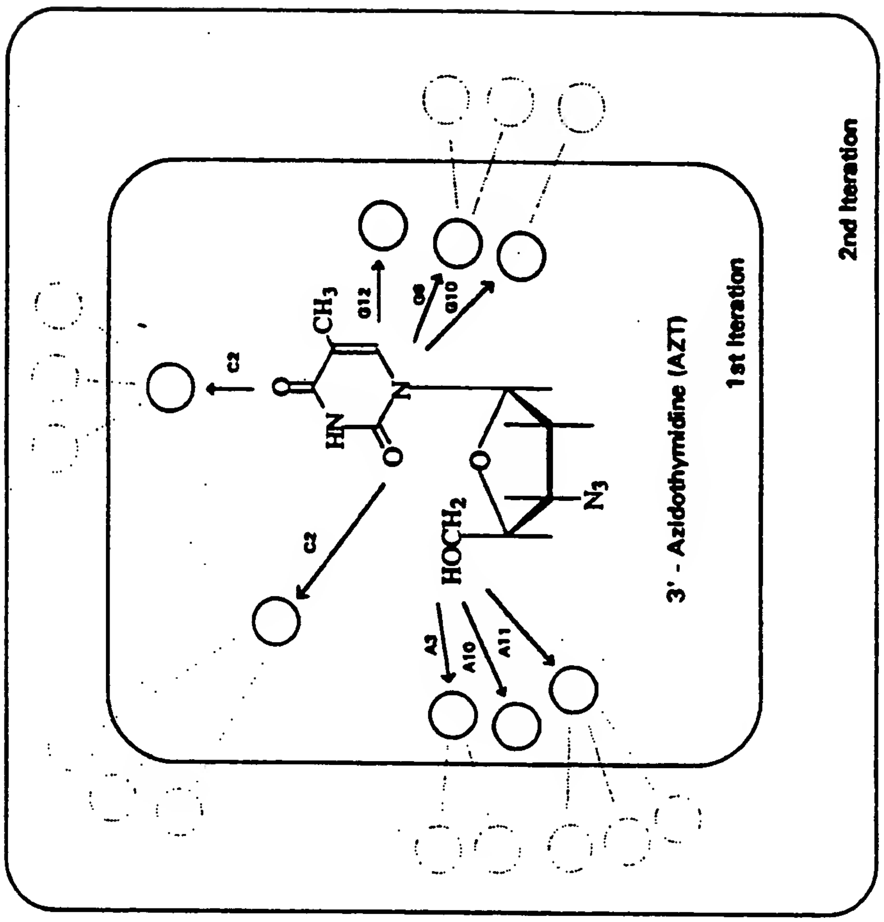
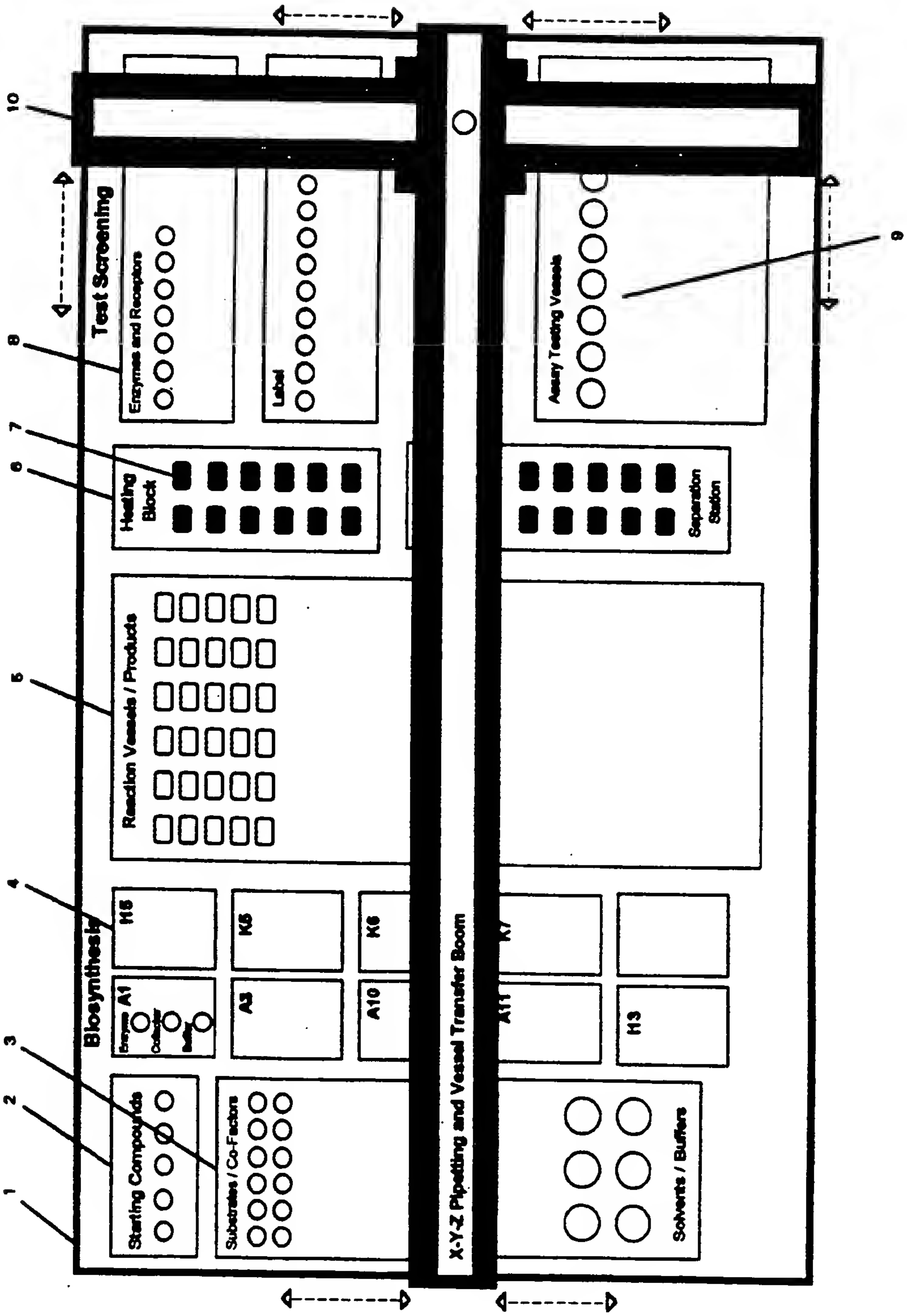

3' - Azidothymidine (AZT)
1st Iteration
2nd Iteration
HOCH$_2$
N$_3$
CH$_3$
HN
C2
G12
G8
G10
A3
A10
A11

FIG. 2



Test Screening
Enzymes and Receptors
Label
Assay Testing Vessels
Heating Block
Separation Station
Reaction Vessels / Products
Biosynthesis
A1
Enzyme
Cofactor
Buffer
H5
K5
K6
K7
A5
A10
A11
H3
Starting Compounds
Substrates / Co-Factors
Solvents / Buffers
X-Y-Z Pipetting and Vessel Transfer Boom
1
2
3
4
5
6
7
8
9
10

FIG. 3

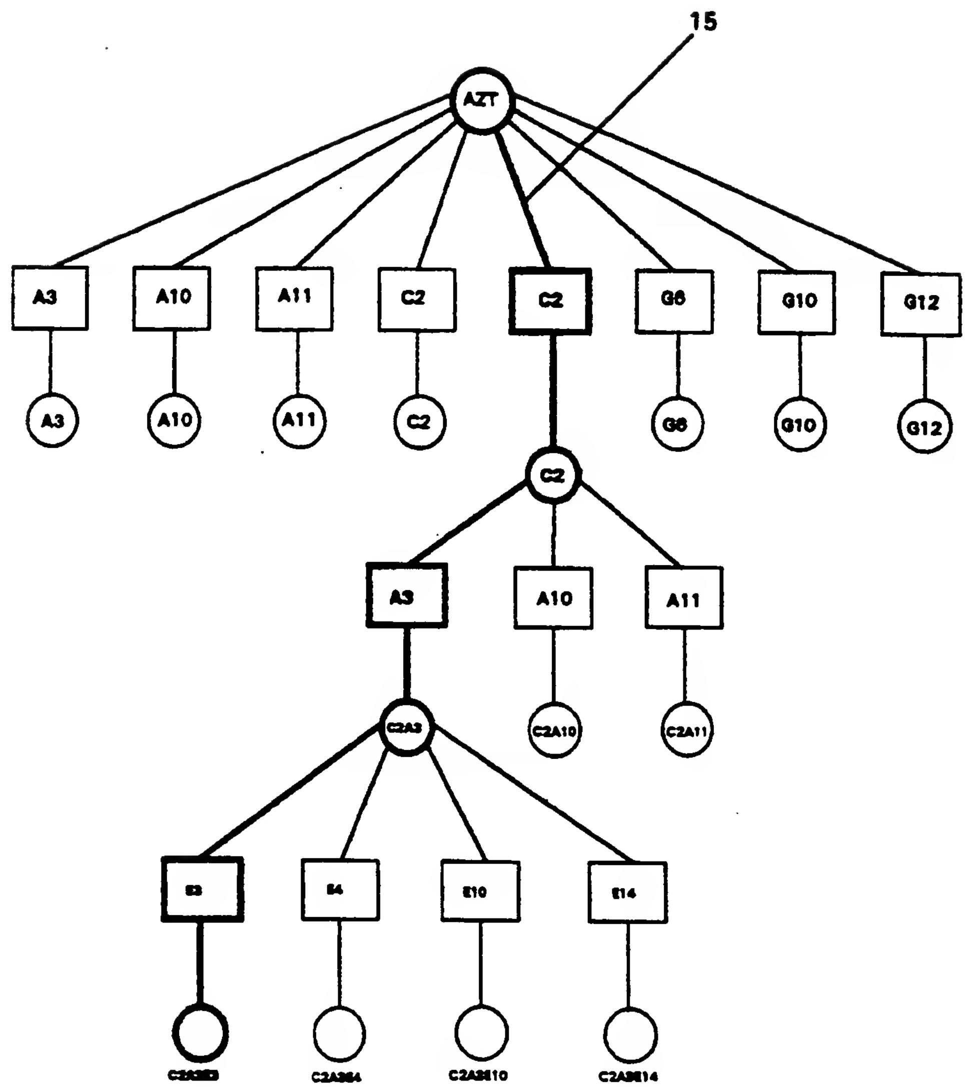

15
AZT
A3
A10
A11
C2
C2
G6
G10
G12
A3
A10
A11
C2
G6
G10
G12
C2
A3
A10
A11
C2A3
C2A10
C2A11
E10
E14
C2A3E10
C2A3E14

FIG. 4a

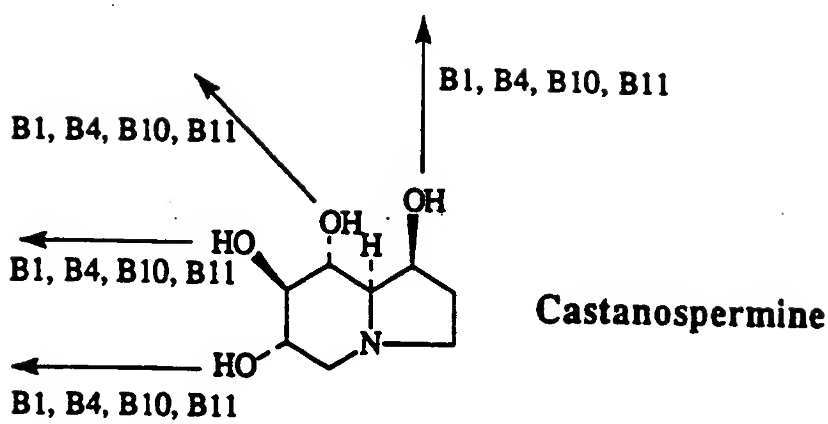
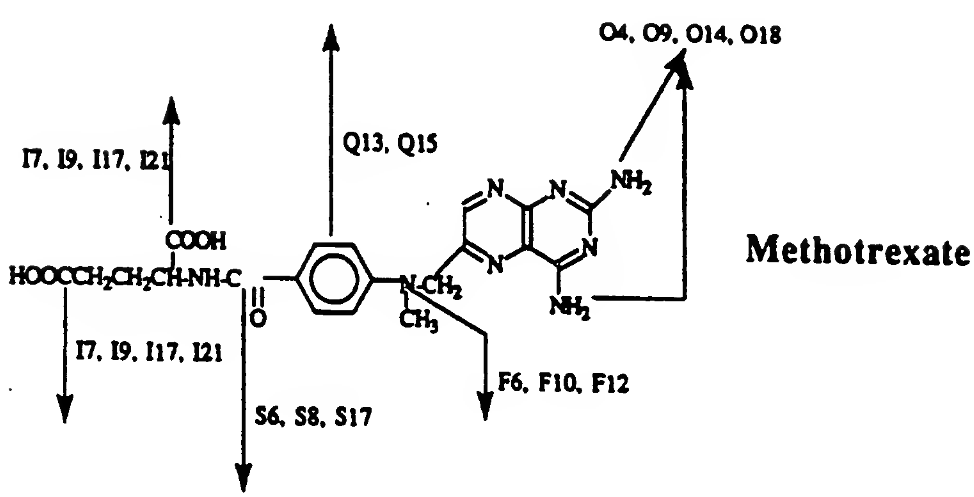

B1, B4, B10, B11
B1, B4, B10, B11
B1, B4, B10, B11
B1, B4, B10, B11
OH
OH
HO
HO
H
N
Castanospermine

FIG. 4b


O4, O9, O14, O18
Q13, Q15
I7, I9, I17, I21
I7, I9, I17, I21
S6, S8, S17
F6, F10, F12
COOH
HOOCCH2CH2CH-NH-C
O
N-CH2
CH3
NH2
NH2
Methotrexate

## A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12P 1/00, C12Q 1/00, G01N 33/50 // C12P 19/32, C12P 17/02

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: G01N, C12P, C12Q, C12M

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPODOC, MEDLINE, BIOSIS, CAS, WPI, PAJ, SCISEARCH, DIALINDEX

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | WO, A1, 9200091 (BIOLIGAND, INC.), 9 January 1992 (09.01.92), page 6, line 28 - page 7, line 20<br>-- | 1-20 |
| A | PROC. NATL. ACAD. SCI., Volume 89, 1992, (05), Ronald N. Zuckermann et al, "Identification of highest-affinity ligands by affinity selection from equimolar peptide mixtures generated by robotic synthesis" page 4505 - page 4509<br>-- | 1-20 |
| A | LETTERS TO NATURE, Volume 354, November 1991, Kit S. Lam et al, "A new type of synthetic peptide library for identifying ligand-binding activity" page 82 - page 84<br>-- | 1-20 |

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

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- "E" erlier document but published on or after the international filing date
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- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed
- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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- "&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 7 December 1994 | 28.12.94 |

| Name and mailing address of the International Searching Authority | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL-2280 HV Rijswijk<br>Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,<br>Fax: (+31-70) 340-3016 | PATRICK ANDERSSON |

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | PROC. NATL. ACAD. SCI., Volume 90, August 1993, Sheila Hobbs De Witt et al, ""Diversomers": an approach to nonpeptide, nonoligomeric chemical diversity" page 6909 - page 6913 | 1-20 |

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| WO-A1- 9200091 | 09/01/92 | AU-A- 8238591 | 23/01/92 |
| | | CA-A- 2086672 | 03/01/92 |
| | | EP-A- 0542770 | 26/05/93 |
| | | JP-T- 6500308 | 13/01/94 |
| | | NZ-A- 238805 | 26/07/94 |